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Activation of mesangial cell signaling cascades in response to mechanical strain

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Activation of mesangial cell signaling cascades in response to mechanical strain.

Background. Mesangial cells (MCs) are constantly exposed to pulsatile stretch and relaxation in their role as architectural support for the glomerulus. There is no cell proliferation in normal glomeruli. In contrast, animal models of increased glomerular capillary pressure are characterized by resident glomerular cell proliferation and elaboration of extracellular matrix (ECM) protein, resulting in glomerulosclerosis. This process can be ameliorated by maneuvers, such as angiotensin converting enzyme inhibition, that reduce glomerular capillary pressure. MCs grown on ECM-coated plates and exposed to cyclic stretch/relaxation proliferate and produce ECM protein, suggesting that this may be a useful *in vitro* model for MC behavior in response to increased physical forces. Previous work has shown induction of c-*fos* in response to application of mechanical strain to MCs, which may induce increases in AP-1 transcription factor activity, which, in turn, may augment ECM protein and transforming growth factor β transcription and cell proliferation. Stimuli that lead to c-*fos* induction pass through mitogen-activated protein kinase (MAPK) pathways. Three MAPK cascades have been characterized in mammalian cells—p44/42 (classic MAPK), the stress-activated protein kinase/Jun terminal kinase (SAPK/JNK) pathway, and p38/HOG—and mechanical strain activates p44/42 and SAPK/JNK in cardiac fibroblasts. However, in contrast to MCs, these cells do not proliferate in response to physical force. Accordingly, we studied activation of the MAPK pathways in MCs exposed to mechanical strain.

Methods. MCs (passages 5 to 10) cultured on type 1 collagen-coated, flexible-bottom plates were exposed to 30, 60, or 120 minutes of cyclic strain (60 cycles/min) by computer-driven generation of vacuums of −14 and −28 kPa, inducing 20% and 29% elongations in the diameter of the surfaces, respectively. Control MCs were grown on coated rigid bottom plates. Proliferation was assessed at 24 hours by ^{3}H-thymidine incorporation. Protein levels (by Western blot) and activity assays for all three kinase cascades were performed at 30, 60, and 120 minutes.

Results. Cyclic strain/relaxation lead to an approximate doubling of ^{3}H-thymidine incorporation at 24 hours ($N = 3$, $P < 0.05$) only in cultures stretched 29%, but not in cultures stretched 20%. At −29% elongation, the increase in ^{3}H-thymidine incorporation was preceded by early activation of MAPK signaling pathways. p44/42 activity increased to a maximum of eightfold greater than control at 60 minutes. p38/HOG activity was not measurable at baseline but was increased markedly at 30 minutes, which was sustained through to 120 minutes. SAPK/JNK activity was present at a very low level in MCs and was not changed by stretch. However, it was markedly increased by sorbitol. In MCs stretched to 20% elongation, lesser increases in p44/42 were seen with a similar time course, whereas no increases in p38/HOG or SAPK could be detected at the time points studied. No increase in any kinase pathway activity was seen at any time in static cultures.

Conclusions. High-pressure cyclic stretch leads to MC proliferation, preceded by marked activation of p44/42 and p38/HOG MAPKs. Cell proliferation is not seen with low-pressure stretch, and there is only modest p44/42 MAPK activation, suggesting that glomerular capillary hypertension may lead to cell proliferation and injury partly through differential activation of kinase cascades.

Key words: glomerular architecture, extracellular matrix protein, glomerulosclerosis, kinase cascade, cell proliferation, hypertension.

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The mesangial cell (MC) is exposed to pulsatile cycles of stretch and relaxation in its role as architectural support for glomerular capillary loops [1]. Indeed, the glomerular microcirculation exhibits vascular pressures greater than those of any other capillary bed [2]. MCs are constantly exposed to such forces, yet little resident glomerular cell proliferation or sclerosis is demonstrable in intact animals. Conversely, intraglomerular hypertension, which would be expected to increase the magnitude of cyclical stretch to which MCs are exposed, has been demonstrated to precede glomerular cell proliferation and the accumulation of extracellular matrix (ECM) protein in several animal models of glomerular sclerosis [3–5]. Moreover, maneuvers that decrease intraglomerular pressure attenuate sclerotic injury in several of the same models, further attesting to the important role of mechanical forces [5–7].

The effects of mechanical forces on MCs *in vitro* have been studied by culturing cells on plates with deformable bottoms and applying vacuum to the well to generate alternating cycles of stretch and relaxation. Akai et al noted increases in cellular calcium entry and total protein

kinase C (PKC) activity within five minutes of the application of stretch to MCs [1], followed by induction of mRNA for the proto-oncogene and AP-1 transcription factor component c-*fos* at 30 minutes [1], and inhibition of PKC blocked this [1]. Several groups have found that this is followed by increases in both collagenous and noncollagenous ECM protein synthesis by 48 hours, the *sine qua non* of sclerotic injury [8, 9]. MC proliferation is also seen, such that approximately twice as many cells are present in stretched cultures after 72 hours when compared with MCs grown under static conditions [2].

Transforming growth factor β1 (TGF-β1) may play a role in the accumulation of ECM in glomeruli [10, 11]. The application of stretch to MCs resulted in up-regulation of TGF-β1 mRNA between 12 and 48 hours [12], supporting the link between stretch, TGF-β1, and sclerosis. Because the TGF-β1 promoter region contains two AP-1 consensus sequences in a positive regulatory region [13, 14] and c-*fos* mediates TGF-β1 autoinduction [15], it is likely that TGF-β1 gene expression in response to stretch is at least partly dependent on AP-1 transcription factor activity.

Cell cycle activity and cell proliferation are controlled by oscillating levels of cyclins. Cyclin D1, the growth factor responsive cyclin important for cell progression through the G1/S point, was increased in response to constant pressure applied to MCs [16]. Activation of classic p44/42 mitogen-activated protein kinase (MAPK) has been demonstrated to increase transcription of the cyclin D1 gene [17].

The link between early events such as PKC activation and induction of transcription of c-*fos*, TGF-β1, and cyclin D1 in response to stretch has been little studied. One group was unable to abrogate the TGF-β1 rise in response to stretch by PKC inhibition, but found the protein tyrosine kinase inhibitors genistein and herbimycin A effective [18]. Others found early increases in extracellular signal-regulated kinase (ERK) and cyclin D1 expression in MCs in response to constant pressure, an effect that could be abrogated by anti-ERK oligonucleotides and tyrosine kinase inhibition [16]. Both PKC and tyrosine kinase receptor signal transduction to the nucleus passes through the MAPK cascade [19]. Platelet-derived growth factor (PDGF), a tyrosine kinase receptor ligand, leads to proliferation and ECM production in MCs via MAPK signaling [20–22]. Consequently, determination of activity of the MAPK cascades in response to stretch would be of considerable importance. Evidence exists that classic MAPK activation occurs in response to cyclical stretch in vascular smooth muscle cells [23] and cardiac myocytes [24–26] and to constant pressure in MCs [16].

There are at least three MAPK cascades in mammalian cells that have been well characterized thus far. Each consists of three protein kinases acting sequentially, a MAP kinase kinase activator, a MAPK activator, and a MAPK [19]. The stress-activated protein kinase/Jun terminal kinase pathway (SAPK/JNK) is activated in response to inflammatory cytokines and mechanical strain [27] in cardiac fibroblasts and leads to phosphorylation and activation of c-Jun. It has been shown to be present in MCs and to respond to endothelin-1 [28]. The p38/HOG pathway is activated by osmolar stresses and heat shock [29, 30], and its downstream targets are as yet undetermined, although phosphorylated p38/HOG is present in the nucleus [31]. p38/HOG has not been studied in MCs but has been shown not to be activated in response to static mechanical strain in cardiac fibroblasts [27].

Accordingly, we sought to determine whether the application of cyclical stretch to MCs led to the activation of canonical MAPK pathways and, if so, which were activated. Kinase activation was compared in two stretch protocols, one of which did not lead to cell proliferation and one which did, to mimic the normal *in vivo* glomerulus and the glomerulus exhibiting capillary hypertension, respectively. Unstretched MCs were also studied.

METHODS

Cell culture

Sprague-Dawley rat MCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (Gibco BRL, Grand Island, NY, USA), streptomycin (100 μg/ml), penicillin (100 U/ml), and 2 mM glutamine at 37°C in 95% air/5% CO_2 as described previously [31]. Experiments were carried out in cells between passages 5 and 10.

Application of stretch/relaxation

Mesangial cells (2×10^6/well) were plated on to six-well plates with either a rigid or flexible bottom coated with bovine type I collagen (Flexcell International Corp., McKeesport, PA, USA). Cells were grown to confluence for 72 hours and were then rendered quiescent by incubation for 24 hours in DMEM with 0.5% fetal calf serum. The cells on the flexible-bottom plated were then exposed to cycles of stretch/relaxation for periods of 30, 60, and 120 minutes by exposure to a cyclic vacuum generated by a computer-driven system (Flexercell Strain Unit 2000; Flexcell). Plates were exposed to continuous cycles of stretch/relaxation, with each cycle being 0.5 seconds of stretch and 0.5 seconds of relaxation for a total of 60 cycles per minute. The low-pressure vacuum was −14 kPa and induced a 20% elongation in the diameter of the surface, whereas the high-pressure vacuum was −28 kPa and induced a 29% elongation in the diameter of the surface.

Effect of mechanical strain on cell proliferation

The incorporation of ^{3}H-thymidine was used as a measure of cell proliferation. MCs were plated in six-well

plates as described earlier here at 5×10^3 cells/cm^2 in DMEM with 20% fetal bovine serum and 100 mg/dl D-glucose. Cells were grown to confluence and were growth arrested and stretched as mentioned earlier here for 24 hours. For the last two hours of this stretch period, 2 μCi/ml ^{3}H-thymidine (DuPont-NEN Life Science Products, Boston, MA, USA) was added to each well. MCs were then washed three times with ice-cold PBS incubated in ice-cold 5% trichloroacetic acid for 25 minutes and were dissolved in 500 μl of 0.2 N NaOH. ^{3}H-thymidine was counted on a Beckman LS 7500 scintillation counter. Protein concentrations were determined in each well, using the Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA, USA). ^{3}H-thymidine incorporation is expressed as counts per minute per μg protein.

Protein isolation

Cellular levels of p44/42, JNK/SAPK, and p38 MAPK proteins were determined in stretched and unstretched control cells at time zero, then at 30 minutes, one hour, and two hours after the application of stretch. Briefly, at the end of each time point, media were removed, and the cells were washed once with ice-cold PBS. PBS was then removed, and cells were harvested under nondenaturing conditions on ice by incubation for five minutes with 0.5 ml 1 × ice-cold cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM egtazic acid, 1% triton, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na orthovanadate, 1 μg/ml leupeptin) and 1 mM phenylmethylsulfonyl fluoride. Cells were then scraped into microcentrifuge tubes on ice and were sonicated four times for five seconds each. After microcentrifugation at 14,000 rpm for 10 minutes at 4°C, the supernatant was transferred to a fresh microcentrifuge tube. Protein concentration was measured with the Bio-Rad assay kit.

Western blotting for MAPK, JNK/SAPK, and p38/HOG

Forty micrograms of sample were then separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electroblotting to a nitrocellulose membrane (Protran, Schleicher and Schuell, Keene, NH, USA), membranes were incubated for three hours at room temperature with 25 ml of blocking buffer [1 × Tris-buffered saline (TBS), 0.1% Tween-20 with 5% wt/vol nonfat dry milk] and then overnight at 4°C with p44/42 MAPK (thr 202/tyr 204) polyclonal antibody (1:1000), SAPK/JNK polyclonal antibody (1:1000), or p38 MAPK polyclonal antibody (1:1000; all New England Biolabs, Beverly, MA, USA) in 10 ml of antibody dilution buffer (1 × TBS, 0.05% Tween-20 with 5% bovine serum albumin; TTBS) with gentle rocking overnight at 4°C. Membranes were then washed three times with TTBS and were then incubated with horseradish peroxidase (HRP)-conjugated antirabbit secondary antibody (1:2000) in 10 ml of blocking buffer for 45 minutes at room temperature. After three further TBS washes, the membrane was incubated with LumiGlo reagent (KPL Inc., Gaithersburg, MD, USA) and was then exposed to x-ray film (X-OMAT; Kodak, Rochester, NY, USA).

MAPK activity assays

Immunoprecipitation. After protein isolation from total cell lysate as mentioned earlier here, 200 μg total protein was then incubated with either p44/42 MAPK (thr 202/tyr 204) monoclonal antibody (1:200) or p38 MAPK antibody (1:100; both New England Biolabs) with gentle rocking overnight at 4°C. Protein sepharose A beads (20 μl of 50% beads) were then added, and the gentle rocking continued for three further hours. Lysate was then microcentrifuged for 30 seconds at 14,000 rpm to recover the beads, and the pellet was washed twice with 0.5 ml of 1× lysis buffer.

p44/42 and p38/HOG MAPK activity assays. Activity of p44/42 and p38 MAPK enzymes was determined in stretched and unstretched control cells at time zero, then at 30 minutes, one hour, and two hours after the application of stretch using the respective MAPK assay kits (New England Biolabs Inc.).

For the kinase assay, after immunoprecipitation, pellets were washed twice with 0.5 ml kinase buffer (25 mM Tris, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na orthovanadate, and 10 mM $MgCl_2$). p44/42 MAPK activity was then performed by suspending the pellet in 50 μl of 1 × kinase buffer, with 200 μM adenosine triphosphate and 2 μg Elk-1 fusion protein, and p38 activity by using 2 μg AP-2 transcription factor (ATF-2) fusion protein as substrate, and a phospho-specific anti-ATF-2 antibody as the primary antibody. After incubation for 30 minutes at 30°C, the reaction was terminated with 25 μM 3 × SDS sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% wt/vol SDS, 30% glycerol, 150 mM dithiothreitol, 0.3% wt/vol bromophenol blue), boiled for five minutes, vortexed, and then microcentrifuged for two minutes. Twenty microliters of sample were then run on an SDS-PAGE gel. After blotting to nitrocellulose, membranes were incubated for three hours at room temperature with 25 ml of blocking buffer (1 × TBS, 0.1% Tween-20 with 5% wt/vol nonfat dry milk) and then overnight at 4°C with phospho-specific anti-Elk-1 (ser 383) antibody (p44/42 MAPK activity) or phospho-specific anti-ATF-2 (thr71) antibody (p38 activity) 1:1000 in 10 ml of antibody dilution buffer (1 × TBS, 0.05% Tween-20 with 5% BSA). Gels were washed three times with TTBS and were then incubated with HRP-conjugated antirabbit secondary antibody (1:2000) for one hour at room temperature. After three further TBS washes, the membrane was incubated with LumiGlo re-

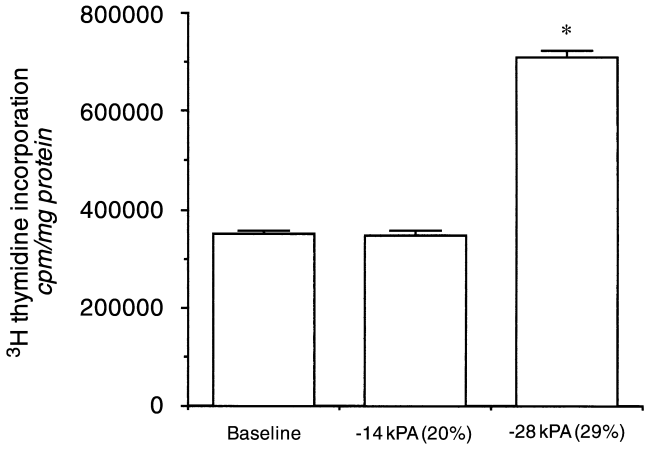

Fig. 1. ^{3}H-thymidine incorporation into stretched mesangial cells (MCs) at 24 hours. Application of −14 kPa mechanical strain (20% elongation) to MCs grown on type 1 collagen-coated deformable plates did not lead to any increase in ^{3}H-thymidine incorporation at 24 hours. Application of −28 kPa mechanical strain (29% elongation) led to significant increases in ^{3}H-thymidine incorporation at 24 hours when compared with either static (baseline) MCs or cells stretched at lower pressure ($N = 3$, $^*P < 0.05$ by unpaired t-test for −29 kPa vs. static or −14 kPa).

agent (KPL Inc.) and was then exposed to x-ray film (X-OMAT; Kodak).

"Pull-down" SAPK assay. After protein isolation as mentioned earlier here, 2 μg of c-Jun fusion protein beads (New England Biolabs) were added to 250 μg of cell lysate protein and were incubated overnight at 4°C. Lysate was then centrifuged for 30 seconds to recover the beads and were washed twice with 1 × lysis buffer as mentioned earlier here. The pellet was then resuspended in kinase buffer and boiled as previously. Twenty microliters of sample were run on a 12% SDS-PAGE gel. Blotting and detection was as mentioned earlier, except that the primary antibody was phospho-specific c-Jun (ser 63) at 1:1000 dilution.

RESULTS

Mechanical strain-induced cell proliferation

Because the aim of this study was to relate kinase activity to cell proliferation, we studied MCs under three conditions. For baseline (static) determinations, cells were grown on nondistensible collagen-1–coated plates. Tritiated thymidine uptake in these cells was compared with MCs subjected to 20% elongation (−14 kPa) or 29% elongation (−28 kPa). As illustrated in Figure 1, 20% elongation did not lead to an increase in ^{3}H-thymidine incorporation at 24 hours when compared with static cultures, whereas, in sharp contrast, the 29% elongation resulted in a doubling of ^{3}H-thymidine incorporation at 24 hours.

p44/42 MAPK expression and activity

We next sought to relate the differences in ^{3}H-thymidine incorporation between the static, 20% elongation, and 29% elongation strain protocols to p44/42 MAPK expression and activity. After 30, 60, and 120 minutes of stretch, cell lysates were subjected to Western blot analysis of p44/42 MAPK expression. Experiments were performed in quadruplicate. We did not observe any change in p44/42 MAPK protein expression in MCs subjected to either 20% (Fig. 2A) or 29% (Fig. 2B) elongation when compared with baseline (static) cells. Cell lysates were then subjected to immunoprecipitation with p44/42 MAPK (thr 202/tyr 204) monoclonal antibody, and p44/42 MAPK activity was measured by Western blot analysis of the phosphorylation of a target Elk-1 fusion protein by the immunoprecipitate using a phospho-specific anti-Elk-1 (ser 383) antibody. When compared with static (baseline) conditions, 20% elongation led to modest twofold increases in p44/42 MAPK activity at 30 and 60 minutes after the initiation of mechanical strain and a threefold increase after 120 minutes. A representative Western blot is shown in Figure 3A. In contrast, 29% elongation led to a sevenfold increase in p44/42 MAPK activity 30 minutes after the initiation of mechanical strain. The marked increase in MAPK activity was sustained at 60 minutes (eightfold) and 120 minutes (fivefold). A representative Western blot is shown in Figure 3B. Relative increases in phosphorylated Elk-1 are shown graphically in Figure 4.

p38/HOG MAPK expression and activity

We next sought to relate the differences in ^{3}H-thymidine incorporation between the static, 20% elongation, and 29% elongation strain protocols to p38 MAPK expression and activity. After 30, 60, and 120 minutes of stretch, cell lysates were subjected to Western blot analysis of p38 MAPK expression. Experiments were performed in quadruplicate. We did not observe any change in p38 MAPK protein expression in MCs subjected to either 20% (Fig. 5A) or 29% (Fig. 5B) elongation when compared with baseline (static) cells. Cell lysates were then subjected to immunoprecipitation with p38 MAPK monoclonal antibody, and p38 MAPK activity was measured by Western blot analysis of the phosphorylation of a target ATF-2 fusion protein by the immunoprecipitate using a phospho-specific anti-ATF-2 antibody. No p38/HOG MAPK activity was detectable at baseline, and the application of 20% elongation to MCs did not result in any measurable p38/HOG MAPK activity. A representative Western blot is shown in Figure 6A. The failure to detect p38/HOG activity following 20% elongation was in sharp contrast to the observations on MCs exposed to 29% elongation. Under these conditions, p38/HOG activity increased markedly 30, 60, and 120 min-

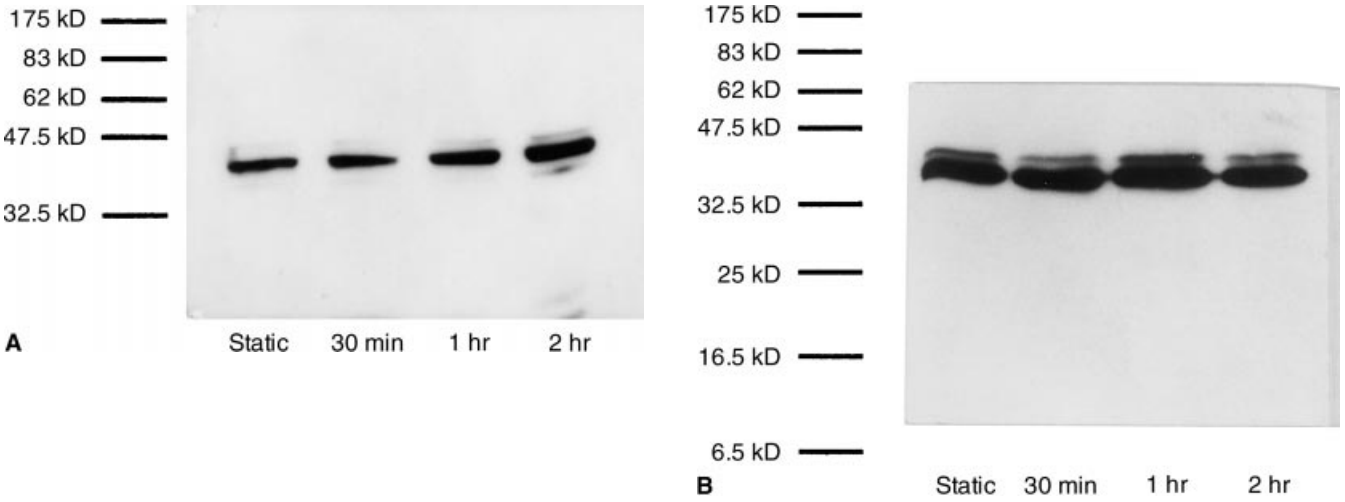

Fig. 2. Representative autoradiographs of p44/42 mitogen activated protein kinase (MAPK) protein expression by Western blot. Bands migrated to the expected 42 and 44 kDa and, consequently, are difficult to separate. Densitometry of the Western blot revealed no significant difference in p44/42 MAPK protein levels at any time point (data not shown). (*A*) Application of −14 kPa mechanical strain (20% elongation) to MCs led to no change in p44/42 MAPK protein expression when compared with static (baseline) MCs. (*B*) Application of −28 kPa mechanical strain (29% elongation) to MCs led to no change in p44/42 MAPK protein expression when compared with static (baseline) MCs.

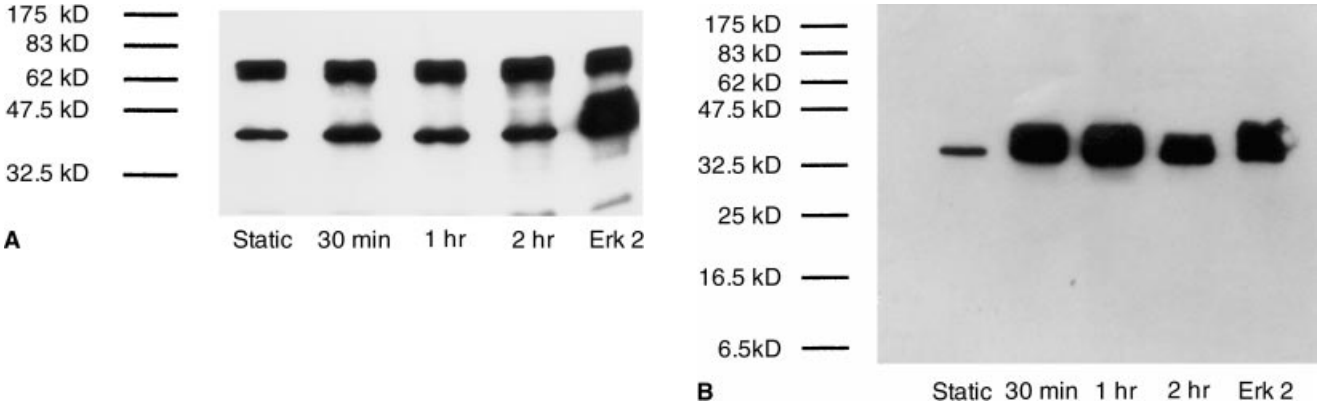

Fig. 3. Representative autoradiographs of p44/42 MAPK activity by Western blot of immunoprecipitates from cell lysates after incubation with an Elk-1 fusion protein. The expected molecular weight of the phosphorylated substrate, Elk-1, is 40 kDa. (*A*) Application of −14 kPa mechanical strain (20% elongation) to MCs led to an increase in p44/42 MAPK activity of twofold at 30 and 60 minutes and rose to a level of threefold at 120 minutes. (*B*) Application of −28 kPa mechanical strain (29% elongation) to MCs led to an increase in p44/42 MAPK activity of sevenfold at 30 minutes, reached a maximum of eightfold at 60 minutes, and was sustained at a level of fivefold at 120 minutes.

utes after the application of stretch. The peak increase in P38/HOG MAPK activity was observed at 30 minutes, and measures declined by 50% at 60 minutes and 75% by 120 minutes. Figure 6B shows a representative Western blot of p38/HOG MAPK activity, and densitometry measures are shown graphically in Figure 7.

SAPK/JNK protein and activity

Finally, we sought to relate the differences in ^{3}H-thymidine incorporation between the static, 20% elongation, and 29% elongation strain protocols to SAPK/JNK expression and activity. After 30, 60, and 120 minutes of stretch, cell lysates were subjected to Western blot analysis of SAPK/JNK expression. Experiments were performed in quadruplicate. We did not observe any change in SAPK/JNK protein expression in MCs subjected to either 20% (Fig. 8A) or 29% (Fig. 8B) elongation when compared with baseline (static) cells. SAPK/JNK activity was measured by Western blot analysis of the phosphorylation of a target c-Jun fusion protein by the cell lysate using a phospho-specific anti-c-Jun antibody for detection. No SAPK/JNK activity was detectable at baseline or after the application of either 20% or 29% elongation to MCs. Representative Western blots including positive controls are shown in Figure 9 A (20%) and B (29%).

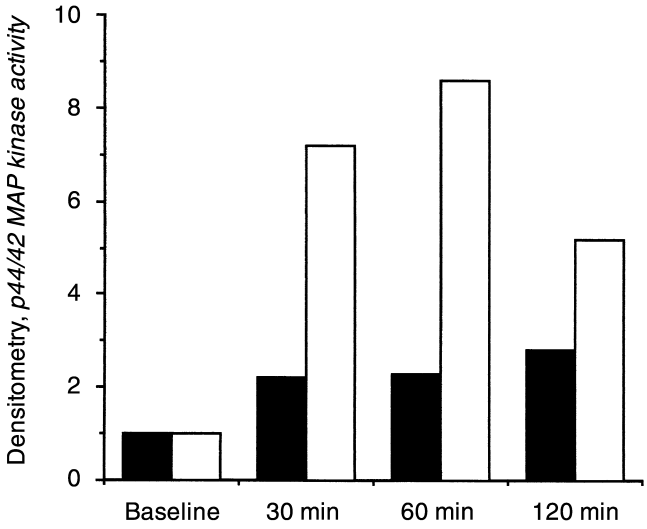

Fig. 4. Densitometry of p44/42 MAPK activity data. Bars show fold increase in p44/42 MAPK condition for MCs stretched at −14 kPa (■, 20% elongation) and −28 kPa (□, 29% elongation) with static (baseline) activity assigned a value of 1. $N = 4$ for each experiment.

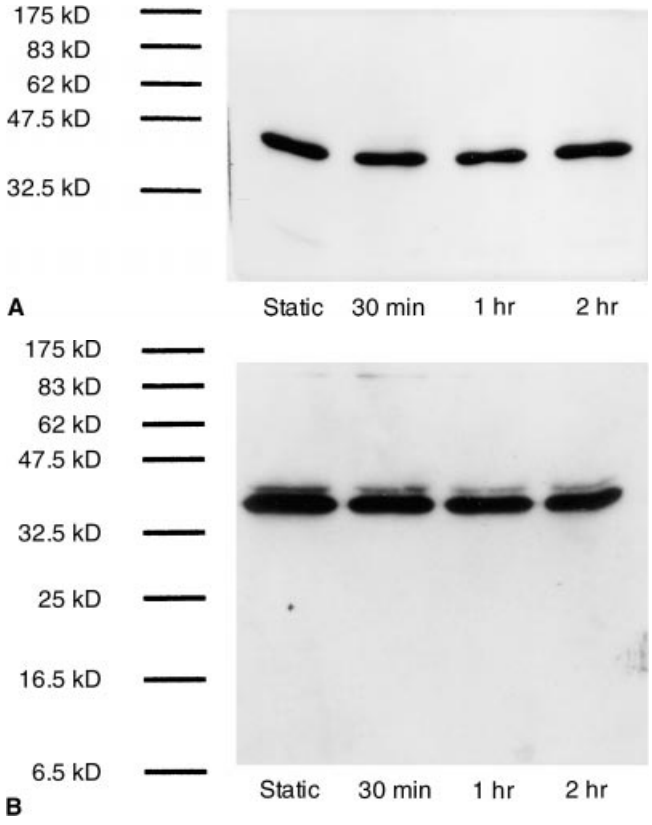

Fig. 5. Representative autoradiographs of p38HOG kinase protein expression by Western blot. The product appears as a 38 kDa band. Densitometry of the Western blot revealed no significant difference in p38/HOG kinase protein levels at any time point (data not shown). (*A*) Application of −14 kPa mechanical strain (20% elongation) to MCs led to no change in p38 HOG kinase protein expression when compared to static (baseline) MCs. (*B*) Application of −28 kPa mechanical strain (29% elongation) to MCs led to no change in p38 HOG kinase protein expression when compared with static (baseline) MCs.

DISCUSSION

Cells exposed to the vasculature are constantly subject to the mechanical stimulus of cyclical stretch and relaxation. Mechanical stresses have been shown to trigger various intracellular events, including increased ECM gene expression and mitogenesis [27]. In the systemic vasculature, this may result in atherosclerotic lesions at areas of high mechanical stress, whereas MC responses may result in glomerulosclerosis [8, 9, 12]. In the normal glomerulus, however, MCs are constantly exposed to the highest pulsatile capillary pressures in the body, and no cell proliferation or sclerosis is observed [32].

Thus far, examination of the events in MCs in response to stress has focused on the elaboration of ECM protein and the control of cell proliferation. Several groups have shown that cyclical stretch leads to MC production of collagenous proteins [2] and fibronectin [18] when compared with cells grown in static cultures. These changes were associated with a rise in TGF-β signaling activity [12, 18, 33]. MC proliferation has also been routinely observed by the same investigators in response to stretch [2, 34].

How these intracellular events might be transduced in response to mechanical strain has more recently been subjected to investigation. Mechanotransduction from exterior to cell interior has been shown to involve at least two pathways. The proliferative effect of stretch is dependent at least partly on the secretion of PDGF in response to the mechanical signal [35]. In MCs, stretch-induced TGF-β expression is tyrosine kinase dependent, suggesting that PDGF may play a role in mechanotransduction of ECM protein as well [18]. The second pathway of importance uses integrin-mediated signaling to transmit mechanical forces. The proliferative effects of mechanical force are, to some extent, matrix dependent, with cells adherent to fibronectin showing the greatest response [36]. This can be inhibited by blocking integrins with RGD peptides [36]. Endothelial cells will remodel focal adhesion sites in response to shear stress [37], so it is quite plausible that cyclic strain might also lead to cytoskeletal rearrangement mediated by integrin-focal adhesion complex interactions. Indeed, static strain resulted in activation of intracellular MAPK signaling pathways in cardiac fibroblasts, an effect clearly demonstrated to be integrin dependent [27].

Subsequent transmission of mechanical stimuli to the nucleus involves the MAPK cascades. Each of the three well-described MAPK cascades consists of three protein kinases acting sequentially, a MAP kinase kinase activator, a MAPK activator, and a MAPK [19]. The classic ERK (p44/42 MAPK) pathway is activated by mitogens and phosphorylates the ternary complex factor Elk-1,

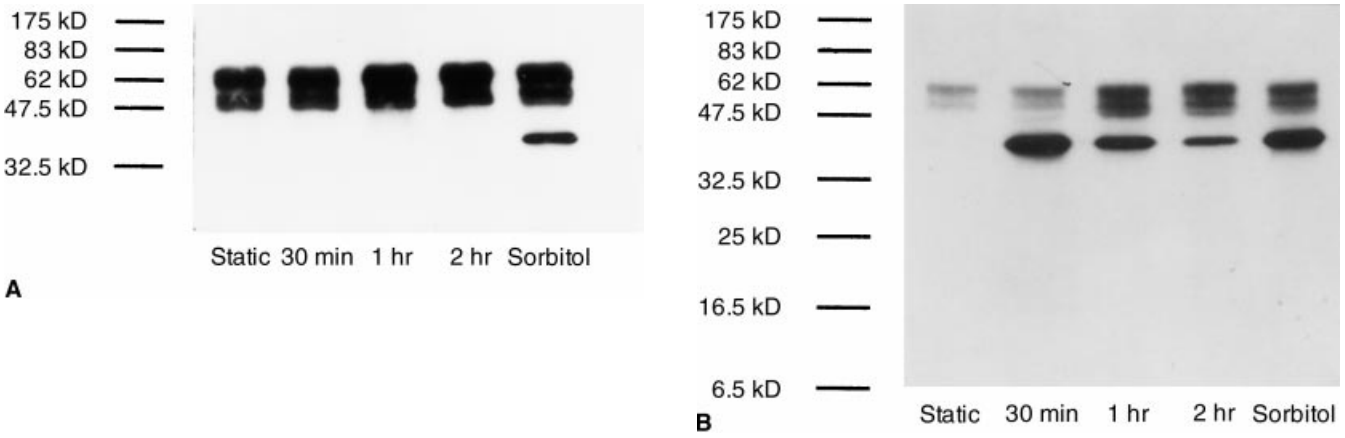

Fig. 6. Representative autoradiographs of p38/HOG kinase activity by Western blot of immunoprecipitates from cell lysates after incubation with an ATF-2 fusion protein. The expected molecular weight of the phosphorylated substrate, ATF-2, is 35 kDa. There was no p38 HOG kinase activity detectable at baseline. (*A*) Application of −14 kPa mechanical strain (20% elongation) to MCs did not result in any p38 HOG kinase activity at any of the time points measured. The right hand lane is a positive control. (*B*) Application of −28 kPa mechanical strain (29% elongation) to MCs led to marked increases in p38 HOG kinase activity at 30 minutes, declining to about 50% of the peak value at 60 minutes and 25% of the peak value at 120 minutes.

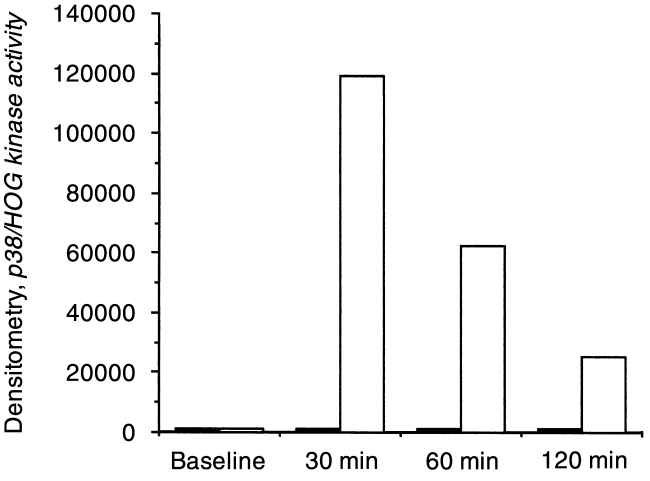

Fig. 7. Densitometry of p38/HOG MAPK activity data. Bars show absolute values for densitometry readings for MCs stretched at −14 kPa (■, 20% elongation) and −28 kPa (□, 29% elongation) with no activity detected in static (baseline) cultures or cells exposed to −14 kPa (20% elongation) stretch at 30, 60, or 120 minutes.

resulting in activation of the ternary complex and transcription of several genes, important among them being the AP-1 component c-fos [38] and the positive cell cycle regulator cyclin D1 [17]. The SAPK/JNK pathway was first described in 1990 [39] and was found to be strongly activated in most cell types by inflammatory cytokines, especially TNF-α and interleukin-1β [40], and by the physical stress of heat shock [40]. In MCs, endothelin-1 activated JNK/SAPK at 15 minutes, an effect that was independent of PKC [28]. Downstream, the JNK/SAPK pathway phosphorylates and activates c-Jun [40]. Phosphorylated c-Jun may then complex with c-Fos to form the AP-1 transcription factor or with ATF-2. The ATF-2/c-Jun dimer binds to promoters containing cAMP-responsive element consensus sequences and may, therefore, activate a different array of genes than AP-1 [41]. The p38/HOG pathway is activated by the physical forces of osmolar stress and heat shock [29, 30]. Although downstream targets of p38/HOG are not as well delineated as for the other two MAPK pathways, p38 does appear to be able to regulate ATF-2 by phosphorylating it on two residues within an N-terminal activation domain [42]. There has been some recent work examining the responses of the canonical MAPK pathways to the physical forces of mechanical stretch and shear stress. Activation of the classic p44/42 pathway has been shown in several cell lines, including endothelia [43], cardiac myocytes [26], and cardiac fibroblasts [27]. Similarly, the JNK/SAPK pathway has been demonstrated to respond to mechanical strain in the same cell lines [26, 27, 43]. p38/HOG did not appear to respond to the application of static strain to cardiac fibroblasts [27]. Cardiac myocytes differ importantly from MCs, however, in that they have not been shown to proliferate in response to mechanical strain [44, 45]. In MCs, activation of p44/42 at one minute was observed recently in response to constant increase in atmospheric pressure, whereas JNK/SAPK did not respond [16, 46].

Clearly, MCs respond to the application of mechanical strain by proliferating and synthesizing matrix proteins [2, 18, 34]. Equally clearly, however, MCs *in vivo* are exposed to constant and relatively high levels of cyclical stretch, and do not proliferate in normal glomeruli. Consequently, there must be some level of mechanical force beyond which a proliferative signal is stimulated. It follows, therefore, that the most appropriate *in vitro* model

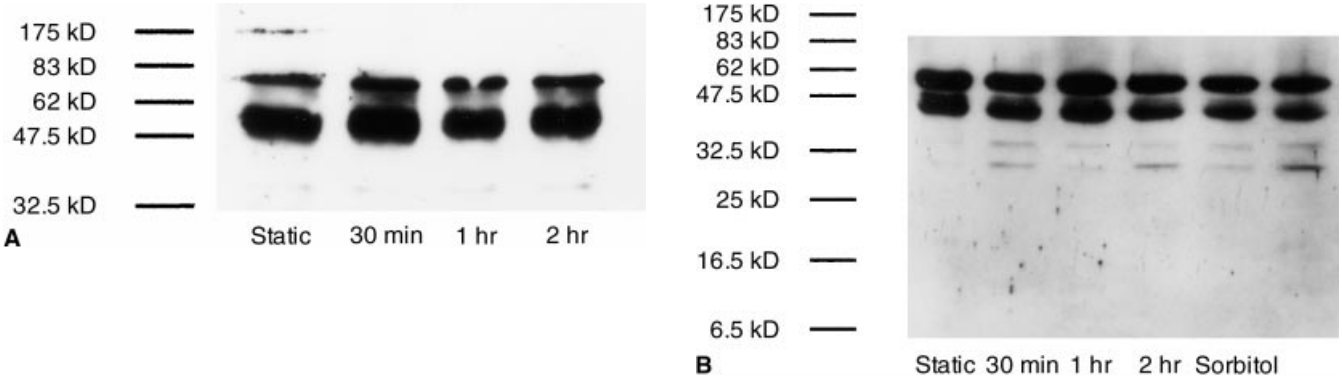

Fig. 8. Representative autoradiographs of SAPK/JNK protein expression by Western blot. Two isoforms exist, with expected product sizes 46 and 54 kDa. Densitometry of the Western blot revealed no significant difference in SAPK/JNK protein levels at any time point (data not shown). (*A*) Application of −14 kPa mechanical strain (20% elongation) to MCs led to no change in SAPK/JNK protein expression when compared to static (baseline) MCs. (*B*) Application of −28 kPa mechanical strain (29% elongation) to MCs led to no change in SAPK/JNK protein expression when compared with static (baseline) MCs.

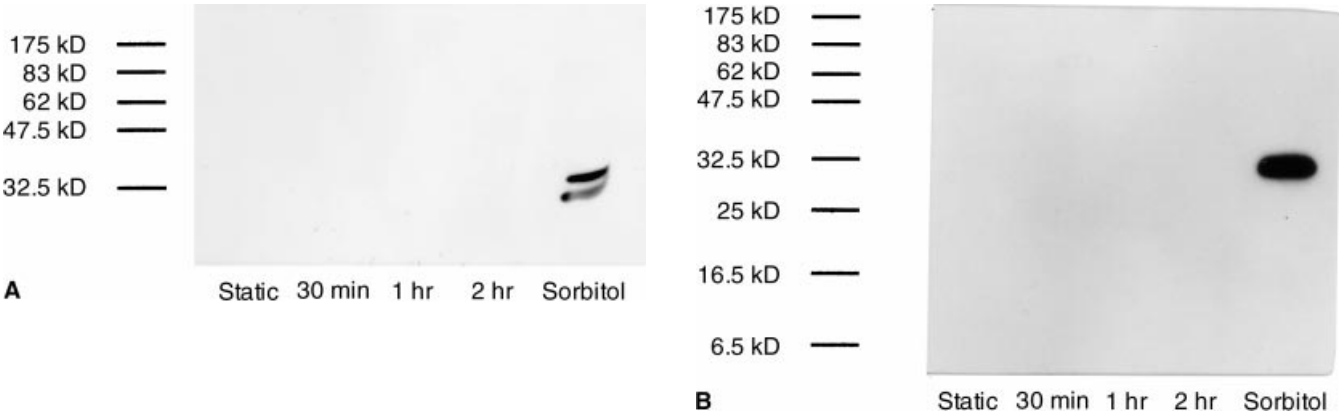

Fig. 9. Representative autoradiographs of SAPK/JNK activity by Western blot of immunoprecipitates from cell lysates. The *in vitro* activity SAPK/JNK was measured using a "pull-down" assay with c-Jun as bait and a phospho-specific anti-c-Jun antibody for detection. The expected molecular weight of the phosphorylated substrate, c-Jun, is 33 kDa. (*A*) Application of −14 kPa mechanical strain (20% elongation) to MCs did not result in any SAPK/JNK activity at any of the time points measured. The right hand lane is a positive (sorbitol) control. (*B*) Application of −28 kPa mechanical strain (29% elongation) to MCs did not result in any SAPK/JNK activity at any of the time points measured. The right hand lane is a positive (sorbitol) control.

for the *in vivo* state would be to compare cell responses with cyclical stretch applied at levels that do and do not lead to proliferation. Accordingly, we examined events in the three well-described MAPK pathways in response to two levels of stretch, one of which resulted in proliferation and one of which did not. As the literature examining cellular responses to physical force has heretofore used static cultures as controls, we also examined kinase cascades in MCs unexposed to physical force. We found clear activation of the classic ERK (p44/42 MAPK) and p38/HOG pathways with the application of 10 minutes of mechanical strain sufficient to stretch cells by 29%, an effect that quickly decayed over one to two hours. In contrast to these observations, JNK/SAPK activity was not increased at these time points. JNK/SAPK protein was present in these cells, however, and sorbitol readily induced activation of the kinase [47]. When cells were stretched at a pressure (−14 kPa) that only leads to 20% elongation, proliferation was not observed at 24 hours. Only modest activation of p44/42 MAPK was observed, although the time course was similar. In contrast to cells stretched at higher pressures, p38/HOG was not activated. Similar to the observations at high-pressure stretch, JNK/SAPK activity was not increased between 30 and 120 minutes. This is in contrast to previous reports in other cell lines, but completely consistent with the results found when mechanical force is applied for 30 minutes to MCs [16, 48]. In a preliminary study, Ishida et al reported earlier activation of SAPK/JNK in MCs in response to physical forces [48], although a more recent publication did not confirm this result [16]. Protein levels of the primary MAPK in each of these cascades (ERK,

p38, and SAPK) were not affected by mechanical stretch at either level. In static cultures, no activation of any pathway was seen at any time point.

There may be cell-specific effects of mechanical strain, as exemplified by the fact that MCs proliferate in response to sufficient physical forces, whereas cardiac myocytes hypertrophy but do not proliferate [44, 45]. In cardiac fibroblasts, static stretch activated p44/42 and JNK/SAPK pathways, but not p38/HOG, an effect that was again matrix dependent [27]. These cells do not proliferate in response to static stretch [27]. Vascular smooth muscle cells proliferate and show classic p44/42 and JNK/SAPK activation in response to stretch, although this is matrix dependent [36]. In our stretched MCs, p44/42 and p38HOG were activated. The uncoupling of p38/HOG and JNK/SAPK, observed by us and Ives et al, is rather unusual [36]. The balance between activation of these pathways in various cell types may underlie differences in outcome in response to the same stimuli. The type and magnitude of the stimulus applied are also likely to be critical. Several methods have been used to mimic *in vivo* hemodynamic force *in vitro*, among them are constant strain [27] and constant pressure [16]. We believe that cyclic stretch would appear to most closely mimic the *in vivo* state, and lower levels of force would likely be the most appropriate control comparator. This is the first report using lower levels of force as a control. Further investigation of these hypotheses awaits examination of how disruption of these pathways might affect MC responses to mechanical strain.

In conclusion, we have shown that cyclical strain to 29% elongation leads to MC proliferation and early activation of the p44/42 and p38/HOG MAPK pathways. A lesser degree of cyclical strain (20% elongation) does not lead to MC proliferation and is associated with a more modest degree of p44/42 MAPK activation. This provides a link between mechanical strain, kinase activation, and cellular proliferation. Further work is necessary to determine the specific mechanisms linking these early kinase activities and the subsequent proliferation of stretched MCs.

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APPENDIX

Abbreviations used in this article are: ATF-2, AP-2 transcription factor; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MCs, mesangial cells; PDGF, platelet-derived growth factor; PKC, protein kinase C; SAPK/JNK, stress-activated protein kinase/Jun terminal kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TGF-β1, transforming growth factor β1.

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